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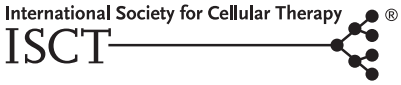

Except for C-C chemokine receptor 7 expression, monocyte-derived dendritic cells from patients with multiple sclerosis are functionally comparable to those of healthy controls

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Abstract

Background aims. Dendritic cell (DC)-based immunotherapy has shown potential to counteract autoimmunity in multiple sclerosis (MS). *Methods.* We compared the phenotype and T-cell stimulatory capacity of *in vitro* generated monocyte-derived DC from MS patients with those from healthy controls. *Results.* Except for an increase in the number of C-C chemokine receptor 7–expressing DC from MS patients, no major differences were found between groups in the expression of maturation-associated membrane markers or in the *in vitro* capacity to stimulate autologous T cells. *Conclusions.* Our observations may pave the way for the development of patient-tailored DC-based vaccination strategies to treat MS.

Key Words: *dendritic cells, immunotherapy, multiple sclerosis, T cell activation*

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (1). The disease is characterized by a heterogeneous disease course and can be either progressive (i.e., chronically-progressive [CP-MS]), or the disease symptoms can be interrupted by periods with partial or complete remission (i.e., relapsing-remitting [RR-MS]). The majority of patients display the latter disease course at disease onset. However, after a moderate level of disability is reached, two thirds of RR-MS patients proceed into a secondary-progressive disease (1). A minority of patients (10–15%) shows a progressive accumulation of disability from disease onset (i.e., primary-progressive [PP-MS]) (1–3). Although MS is considered to be a predominantly T-cell-mediated autoimmune disease, recent findings from several groups suggest that innate immune cells, such as dendritic cells (DCs), control and direct the autoreactive immune response.

DCs are a highly specialized subset of white blood cells that bridge innate immune functions with the induction of adaptive immunity. They orchestrate the outcome of immune responses through a combination of incoming signals. DCs capture, process and present (self-)antigens (i.e., signal 1) in combination with costimulatory molecules (i.e., signal 2) to naive T cells. Upon encounter of so-called danger signals, DCs undergo a complex maturation process that includes upregulated expression of co-stimulatory markers; low expression levels of these molecules are representative of steady-state conditions. In the absence of signal 2, T cells undergo anergy or deletion, resulting in T cell tolerance (4). Furthermore, a new class of pathways delivering tolerogenic signals (i.e., co-inhibitory pathways) has been identified and may be of importance in the induction of Tregs (5). Finally, by secreting a number of cytokines (i.e., signal 3), DCs direct naive T cells to differentiate into various T helper (Th) subsets (4,6,7).

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Table I. Clinical details of the study population.

Code patients	Gender	Age	MS type	EDSS score	Code healthy controls	Gender	Age
UPN 001	F	46	RR	2.5	UPN 036	M	56
UPN 002	F	54	CP	4	UPN 037	M	53
UPN 003	F	46	CP	6.5	UPN 038	F	51
UPN 004	F	39	RR	4	UPN 039	F	56
UPN 005	F	62	CP	6.5	UPN 040	F	48
UPN 006	M	62	CP	6.5	UPN 041	M	46
UPN 007	M	59	CP	7.5	UPN 042	M	44
UPN 008	F	47	RR	2	UPN 043	F	53
UPN 009	F	51	CP	5	UPN 044	F	47
UPN 010	M	48	CP	6.5	UPN 045	F	44
UPN 011	M	52	CP	7.5	UPN 046	F	54
UPN 012	M	56	CP	5.5	UPN 047	F	56
UPN 013	M	44	CP	7	UPN 048	M	55
UPN 014	F	51	CP	6.5	UPN 049	F	48
UPN 015	M	46	CP	6.5	UPN 050	F	23
UPN 016	M	52	CP	6.5	UPN 051	F	44
UPN 017	F	54	RR	2.5	UPN 052	M	38
UPN 018	F	22	RR	0	UPN 053	F	23
UPN 019	F	52	RR	1.5	UPN 054	M	54
UPN 020	F	38	RR	0	UPN 055	M	54
UPN 021	M	43	CP	8	UPN 056	M	55
UPN 022	M	68	CP	6.5	UPN 057	F	38
UPN 023	F	43	CP	8	UPN 058	F	24
UPN 024	F	53	RR	6.5	UPN 059	M	39
UPN 025	F	63	CP	8	UPN 060	F	31
UPN 026	M	72	CP	7.5	UPN 061	M	72
UPN 027	F	74	CP	8	UPN 062	F	29
UPN 028	M	60	CP	8.5	UPN 063	F	66
UPN 029	M	55	CP	5	UPN 064	F	38
UPN 030	M	55	CP	5			
UPN 031	F	28	RR	1			
UPN 032	F	43	RR	4			
UPN 033	M	53	CP	5.5			
UPN 034	F	54	CP	3.5			
UPN 035	M	52	CP	5			
	M/F: 16/19	Median: 52 Range: 22–74	RR/CP: 10/25	Median: 5 Range: 0–8.5		M/F 11/18	Median: 48 Range: 23–72

EDSS, expanded disability status scale; F, female; M, male; UPN, unique patient number.

This so-called T cell polarization will define the regulatory or effector function of stimulated T cells (8,9) and underscores the crucial role of DCs in directing the T cell balance. If the expression of one of these signals is impaired or dysfunctional, the outcome of the T cell response will likely be affected.

Given their central role in maintaining the balance between immunity and tolerance, *ex vivo*–generated immature and/or tolerogenic monocyte–derived DCs have been proposed as a tool to reestablish tolerance in autoimmune disorders mediated by self-reactive T cells that escaped thymic deletion (10,11). In this context, a milestone study by Dhodapkar *et al.* (12) has previously demonstrated that a single injection of antigen-loaded immature DCs was able to inhibit antigen-specific T cell induction in healthy subjects. More studies have revealed the safety and potential clinical benefit of therapeutic vaccination with tolerogenic DCs in patients (13,14), but to date information with regard to the *in vitro* generation and function of DCs from patients with MS is limited. In addition, we and others have demonstrated that circulating myeloid and plasmacytoid DCs of MS patients display pro-inflammatory characteristics compared with steady-state DCs from healthy controls (15–18). In this study, monocyte-derived DCs from MS patients were compared with those from controls with regard to membrane phenotype and autologous T cell stimulatory capacity.

Methods

MS patients, diagnosed according to McDonald criteria (19), were recruited by the Departments of Neurology of the National MS Center (Melsbroek,

Belgium) and of the Antwerp University Hospital (Edegem, Belgium). Thirty-five patients (16 men and 19 women) with a median age of 52 (range: 22−74 years), a median Expanded Disability Status Scale (EDSS) score of 5 (range: 0−8.5) and without MS-related medication at the time of sampling were included (Table I). Twenty-nine age- and gender-matched healthy individuals were included as control subjects. All subjects gave informed consent in accordance with the declaration of Helsinki, and the study protocol was approved by the local ethics committees of the National MS Center and of the Antwerp University Hospital.

CD14+ monocytes were isolated from heparinized peripheral blood as described previously (8). The CD14-depleted cell fraction (i.e., peripheral blood lymphocytes [PBLs]) was cryopreserved and stored at −80°C. To generate monocyte-derived DCs (moDCs), $1-2 \times 10^6$ CD14+ cells/mL were differentiated in the presence of 25 ng/mL interleukin (IL)-4 and 17.5 ng/mL granulocyte macrophage colony-stimulating factor. On day 6, DCs were stimulated for 24 hours by adding a cocktail of pro-inflammatory cytokines consisting of 100 U/mL IL-1, 500 U/mL IL-6, 2.5 ng/mL tumour necrosis factor (TNF)-α and 10^{-7} M prostaglandin E_2 (PGE_2, Prostin E_2) for the generation of mature DCs (mDCs) or left untreated for the generation of immature DCs (iDCs). On day 7, immunophenotyping of DCs was preformed using the following murine anti-human antibodies: anti-C-C chemokine receptor (CCR)7, anti-CD83, anti-CD80, anti-CD86, anti-DC-SIGN and anti-HLA-DR. For analytical flow cytometry, at least 10^4 events were analyzed using a BD FACScan (BD Biosciences, Erembodegem, Belgium).

For T cell activation, PBLs were cultured in presence or absence of iDCs or mDCs. After 7 days, induction of CD4+CD25+ T cells was determined using the following murine anti-human antibodies: anti-CD8, anti-CD3, anti-CD4 and anti-CD25. Dead cells were excluded using a LIVE/DEAD fixable Dead Cell Stain kit. At least 5×10^4 CD3+CD4+CD8- lymphocytes were analyzed on a Cyflow ML flow cytometer (Partec GmbH, Münster, Germany). For myelin-specific T cell induction, PBLs were stimulated with a pool of myelin-derived peptides (i.e., myelin oligodendrocyte glycoprotein $[MOG]_{1-22}$, MOG_{34-56}, MOG_{64-86}, MOG_{74-96} and myelin basic protein $(MBP)_{84-102}$ and $MBP_{143-168}$) (20) in presence or absence of iDCs or mDCs. After 5 days, interferon (IFN)-γ secretion was evaluated by IFN-γ enzyme-linked immunosorbent spot assay (ELISpot) following antigenic restimulation. Non-restimulated PBLs served as a negative control. For the definition of a MOG/MBP responder, the following criteria were followed: per 10^6 PBLs stimulated with mature moDCs, the mean antigen-specific spot count must be greater than or equal to 20 spots and at least three times as high as background.

Comparisons were validated using a paired *t*-test or one-way analysis of variance for observations within one subject and using an unpaired *t*-test for independent observations. Odds ratios were calculated with a Fisher's exact test. All statistics were calculated using GraphPad version 5 software (Prism, La Jolla, CA, USA). A P value <0.05 was considered statistically significant (*) and a P value <0.01 (**) and <0.001 (***) as highly statistically significant.

Results

Higher expression of CCR7 in mo DCs from MS patients

MoDCs of MS patients and of healthy controls were phenotypically characterized using multiparametric flow cytometry. A modest but statistically significant increased relative proportion of iDCs expressing the costimulatory molecule CD86 and the antigen-presenting molecule HLA-DR was found in MS patients (Table II), whereas no differences for the expression

Table II. Expression of membrane markers of DCs of MS patients compared with those of healthy control subjects.

		HC			MS			*P* value HC vs. MS	
		iDC	mDC		iDC	mDC		iDC	mDC
DC-SIGN	MFI	287	228	***	280	191	***	ns	ns
	%	92	88	***	89	80	***	ns	<0.01
HLA-DR	MFI	99	165	***	127	184	**	ns	ns
	%	89	95	*	97	96		<0.05	ns
CD80	MFI	13	29	***	16	33	***	ns	ns
	%	26	58	***	26	54	***	ns	ns
CD83	MFI	13	45	***	18	50	***	ns	ns
	%	2	33	***	5	32	***	ns	ns
CD86	MFI	214	569	***	229	526	***	ns	ns
	%	97	99	***	98	99	*	<0.05	ns
CCR7	MFI	15	33	***	22	35	***	ns	ns
	%	1	10	**	9	16	*	<0.05	<0.05

Immature monocyte-derived DCs were obtained after differentiation of CD14+ cells with IL-4 and granulocyte macrophage colony-stimulating factor. On day 6 of cell culture, DCs were activated with a cocktail of pro-inflammatory cytokines (i.e., mDC) or left untreated (i.e., iDC). The DC phenotype was analyzed on day 7 by multiparametric flow cytometry. Results are shown as mean value of 17 MS patients and 16 HCs. P values comparing the difference within one group (iDC vs. mDC) are indicated by an asterisk and validated using a paired *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). P values comparing DCs of patients with those of controls are shown in the last column. These observations were validated using an unpaired *t*-test.

CCR, C-C chemokine receptor; CD, cluster of differentiation; DC-SIGN, DC-specific intercellular adhesion molecule-3-grabbing non-integrin; HCs, healthy control subjects; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; ns, not significant.

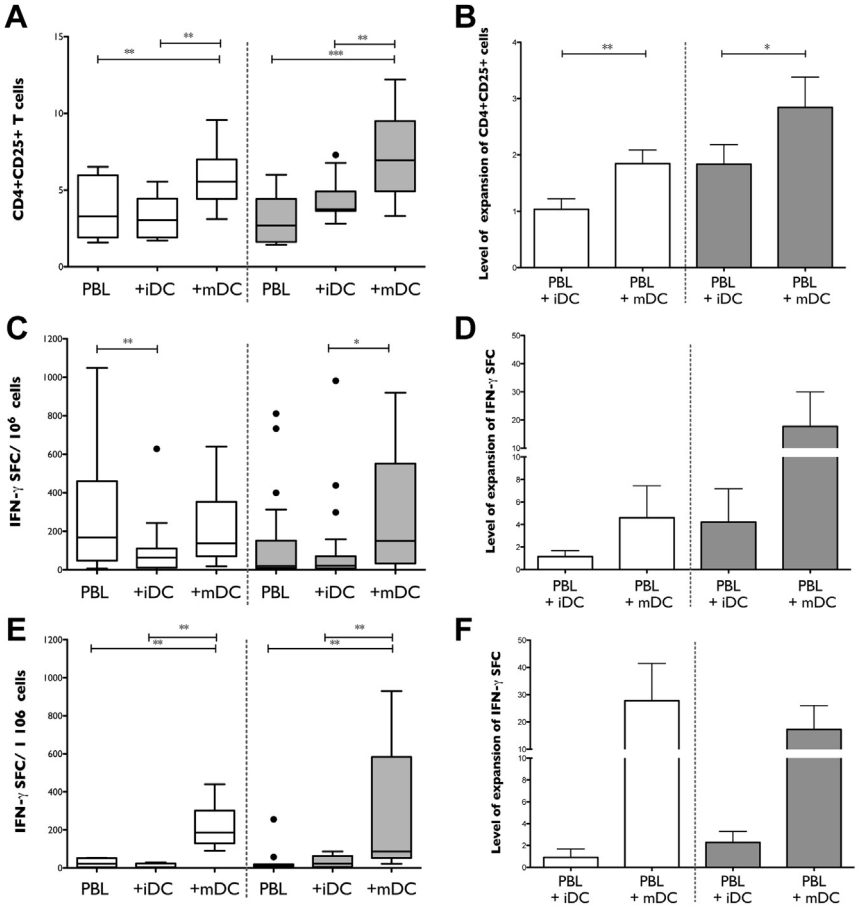

Figure 1. T cell stimulatory capacity of monocyte-derived DCs from MS patients compared with moDCs from healthy controls. (A) Flow cytometric determination of activated T cell frequency as indicated by CD4+CD25+ expression. Autologous PBLs were analyzed after a 7-day DC/T cell co-culture. (B) The DC-mediated expansion of PBLs was calculated by dividing the frequency CD4+CD25+ following DC stimulation with the baseline CD4+CD25+ frequency (i.e., PBLs alone). Results are shown for 11 MS patients (filled bars) and 8 healthy control subjects (open bars). (C) Detection of DC-mediated IFN-γ secretion by PBLs using ELISpot after a 5-day culture of autologous PBLs in presence or absence of iDCs or mDCs. (D) The DC-mediated expansion of the IFN-γ secretion was calculated by dividing the number of spot-forming cells (SFC) following DC stimulation with the baseline number of SFC (i.e., PBL alone). Results are shown for 23 MS patients (filled bars) and 22 healthy controls (open bars). (E) Detection of myelin-specific IFN-γ secretion by ELISpot analysis. Following stimulation of autologous PBLs with a pool of myelin-derived peptides in presence or absence of iDCs or mDCs, PBLs were restimulated using a myelin-derived peptide pool. (F) The DC-mediated expansion of the myelin-specific IFN-γ secretion was calculated by dividing the number of spot-forming cells (SFC) following DC stimulation with the baseline number of SFC (i.e., PBLs alone). Results are shown as the number of antigen-induced SFC corrected for the negative control for 11 antigen-responding MS patients (filled bars) and for 6 antigen-responding healthy control subjects (open bars). All results are expressed as mean + standard error of mean (SEM) or as Tukey whiskers boxplots. Comparisons were validated using a paired *t*-test or one-way analysis of variance for observations within one subject and using an unpaired *t*-test for independent observations. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

of CD83, CD80 and DC-SIGN could be detected compared with immature moDC from healthy controls. Following stimulation with a pro-inflammatory cytokine cocktail initiating the maturation process of moDCs, similar numbers of moDC expressing CD80, CD83, CD86 and HLA-DR were found in MS patients and healthy controls. No differences were observed between RR-MS and CP-MS patients (data not shown). However, DC SIGN−positive mature moDCs were significantly lower in RR-MS but not in CP-MS patients compared with healthy controls. Interestingly, both immature and mature moDCs from RR-MS but not CP-MS patients are characterized by a significantly higher proportion of CCR7-expressing cells compared with those from controls (Table II).

Autologous DC-mediated T cell stimulation reveals no differences between MS patients and healthy controls

First, the overall autologous T cell stimulatory capacity of *in vitro*−generated DCs from MS patients and age- and gender-matched control subjects was evaluated. For this, autologous PBLs of MS patients and healthy control subjects were stimulated for 7 days with immature or mature moDCs. Next, the frequency of

CD4+ T cells expressing the activation marker CD25 was determined as a measure of T cell activation. Our results show that expression of the activation marker CD25 is significantly induced after stimulation with mDCs (Figure 1A), indicating expansion of a significantly greater proportion of these cells compared with stimulation with iDCs (Figure 1B). Importantly, DCs of MS patients display a similar capacity to induce T cell activation to that of controls. In addition, no difference in the number of IFN-γ spot-forming cells was observed between MS patients and healthy control subjects (Figure 1C and 1D).

MoDCs of MS patients and healthy controls induce similar myelin-specific IFN-γ secretion

Finally, we investigated the antigen-specific T cell stimulatory capacity of DCs from MS patients and healthy control subjects. PBLs were stimulated with a pool of myelin-derived peptides in the presence or absence of iDCs and mDCs in an autologous context. Subsequently, myelin-specific IFN-γ secretion was determined by IFN-γ ELISpot following antigen restimulation. No significant differences in myelin-specific T cell-stimulatory capacity between DCs of MS patients and of control subjects after antigenic restimulation could be detected (Figure 1E and 1F). Of interest, odds ratio showed no significant difference in the number of MOG/MBP responders between MS patients and healthy control subjects: 11 of 32 MS patients (27%) and 6 of 20 healthy control subjects (30%) displayed a defined myelin-specific response.

Discussion

Until now, conflicting data have been reported regarding the membrane phenotype of monocyte-derived DCs from MS patients. Although some authors have described a reduced expression of CD83 (21) and a decreased frequency of CD86-positive DCs (22), others reported no differences in MS patients (21,23,24). In contrast, we report a modest, but statistically significant, increased relative proportion of iDCs from MS patients expressing the costimulatory molecule CD86 and the antigen-presenting molecule HLA-DR. However, no differences were found between the expression levels of DC-characteristic membrane markers per cell between patients and control subjects. Although it remains to be established if the observed difference in the expression profile of costimulatory markers of *in vitro* generated DCs between MS patients and controls is of physiological relevance, discrepancies between different studies could also be attributed to treatment effects (21,23,24) or genetic variability (25).

Furthermore, and to our knowledge for the first time, we observed a significantly higher proportion of CCR7-expressing DCs from MS patients compared with control subjects. Increased CCR7 expression may result in improved migration to the lymph nodes, which would be required for therapeutic vaccination. Indeed, Verdijk *et al.* have previously shown that less than 4% of migratory DCs are sufficient to induce antigen-specific immunologic responses in the draining lymph nodes (26). Additionally, because the process of DC maturation involves, among others actions, redistribution in the expression of chemokine receptors, this suggests a more activated state of MS-derived DCs. This may result in stronger T cell activation by a CCR7-mediated increase of the cell surface and life span of DCs and may be detrimental in a clinical setting where induction of tolerance is the aim (27).

To determine if this was the case, we evaluated for the first time the autologous T cell stimulatory capacity of monocyte-derived DCs generated from MS patients and demonstrate no significant differences with those of control subjects in two independent read-out methods. Other authors demonstrated no effect on proliferation (21,28), but increased secretion of IFN-γ (28), in an allogeneic mixed lymphocyte reaction following stimulation with DCs from MS patients compared with control subjects.

Autoreactive T cell responses against myelin antigens are considered to play a primary role in the pathogenesis of MS and have been extensively studied in patients with MS and control subjects (20,29–34). Here, we analyzed T cell reactivity to a panel of MOG- and MBP-derived synthetic peptides that were previously demonstrated to be immunodominant (20,35–37) and not HLA-DR restricted (20,32,38). Furthermore, these peptides correspond to epitopes shown to be encephalitogenic in several strains of rodents and non-human primates (39). No significant differences could be detected by IFN-γ ELISpot between autologous myelin-specific T cell stimulatory capacity of DCs from MS patients and control subjects, following antigenic restimulation, in agreement with current scientific evidence (20,29–34).

In conclusion, monocyte-derived DCs from MS patients are functionally comparable to those from control subjects. For this, our data suggest no contraindications to use iDCs of MS patients and that they thus may be suitable for tolerance-inducing immunotherapies (40,41). Nevertheless, a major concern may relate to the risk of *in vivo* activation, particularly in response to any underlying inflammatory micro-environment, and this has tempered the use of tolerogenic DC *in vivo* by the need to preserve the tolerogenic phenotype. For this, strategies to harness the tolerogenic capacity of DCs have been

developed. *In vitro* treatment of monocyte-derived DCs with IFN-ß, glatiramer acetate (24,28), vitamin D3 (23) and IL-10 (28) are capable of modulating DC phenotype and/or function, making these molecules attractive biological agents for future studies.

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